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Amino-Terminal Processing of MIP-1β/CCL4 by CD26/Dipeptidyl-Peptidase IV

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Abstract CD26 is a membrane-bound ectopeptidase with dipeptidyl peptidase IV (DPPIV) activity that has diverse functional properties in T cell physiology and in regulation of bioactive peptides. We have previously reported that activated human peripheral lymphocytes (PBL) secrete an amino-terminal truncated form of macrophage inflammatory protein (MIP)-1β/(3–69) with novel functional specificity for CCR1, 2, and 5. In this report, we show that the full length MIP-1β is processed by CD26/DPPIV to the truncated form and that cleavage can be blocked by DPPIV inhibitory peptides derived from HIV Tat(1–9) or the thromboxane A2 receptor, TAX2-R(1–9). Addition of Tat(1–9) or TAX2-R(1–9) peptides to PBL cultures partially blocks endogenous MIP-1β processing. The kinetics of conversion of MIP-1β from intact to MIP-1β(3–69) in activated PBLs correlates with cell surface expression of CD26. Our results suggest that NH_2-terminal processing of MIP-1β and possibly other chemokines may depend on the balance between CD26/DPPIV enzymatic activity and cellular and viral proteins that modulate enzyme function. J. Cell. Biochem. 92: 53–64, 2004.

Key words: MIP-1β; CD26/DPPIV; HIV-1 Tat

CD26 is a widely distributed 110 kDa transmembrane protein with known dipeptidyl peptidase IV (DPPIV) activity in its extracellular domain. This multifunctional molecule plays different roles in immune responses. CD26 is associated with adenosine deaminase (ADA) on the cell surface and is able to transduce costimulatory signals in T cells [Kameoka et al., 1993]. The coexpression of surface ADA and CD26 in activated T cells is triggered by anti-CD3 Abs plus IL2 or phorbol esters [Martin et al., 1995]. Within human $CD4^+$ lymphocytes, CD26 is preferentially expressed on the memory/helper subset. This unique population of $CD4^+CD26^+$ cells responds to recall antigens and facilitates B-cell immunoglobulin production and maturation of MHC-restricted cytotoxic T cells. Cell surface expression of CD26 is strongly associated with human Th1 responses [Torimoto et al., 1991; Scheel-Toellner et al., 1995] and is up regulated following mitogenic, antigenic, or cytokine (IL2) stimulation of T cells. It has been recently reported that in the presence of the CXCR4 ligand, SDF-1α, CD26 is cointernalized with CXCR4 in human lymphocytes, suggesting a role for CD26/CXCR4 complexes in modulation of SDF-1α/CXCL12 chemotaxis and antiviral capacity [Herrera et al., 2001].

The expression of CD26 can affect viral replication, and viral proteins are reported to modify CD26 activity. Susceptibility of a T cell line to HIV-1 infection correlates with CD26 expression [Oravecz et al., 1995]. Association of ADA with CD26 is inhibited in the presence of

Abbreviations used: MIP, macrophage inflammatory protein; HIV-1, human immunodeficiency virus-type 1; IL, interleukin; PHA, phytohemagglutinin; PBL, peripheral blood lymphocyte; MCP, macrophage chemoattractant protein; GCP, granulocyte chemotactic protein; Mig, monokine induced by interferon-γ; GRO, growth-related oncogene; IP-10, interferon-γ-inducible protein-10; DTT, dithiothreitol; Th, T helper.

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gp120 [Valenzuela et al., 1997]. HIV-1 proteins Tat and gp120 directly bind CD26 [Gutheil et al., 1994] and the full length Tat protein and a nine amino-acid NH_2-terminal peptide inhibit enzymatic DPPIV activity of CD26 [Smith et al., 1998]. A nine amino acid Tat-homologous peptide from the thromboxane receptor (TXA2-R) expressed on monocyte/macrophages also inhibits DPPIV, suggesting that TXA2-R may function to control enzyme activity on the cell surface.

It is well known that CD26 has a unique aminopeptidase activity capable of cleaving N-terminal dipeptides from polypeptides with either proline or alanine residue in the penultimate position. Despite the seemingly minimal loss of two NH_2-terminal amino acids following truncation by DPPIV, many cleaved regulatory peptides and proteins are either totally inactivated or show altered receptor targeted activities. Important DPPIV substrates include certain neuropeptides (such as neuropeptide Y or endomorphin), circulating hormones (peptide YY, growth hormone-releasing hormone, glucagon-like peptides (GLP)-1 and -2, gastric inhibitory polypeptide), and chemokines [Oravecz et al., 1997; Boonacker and Van Noorden, 2003].

Chemokines are a large group of secreted proteins that act as cell-type selective chemoattractants and some chemokines act as anti-HIV-1 factors. They can be divided into four subfamilies. The two most important subfamilies are the CXC and CC chemokines, which differ in the spacing of the first two cysteine residues. Chemokines exert anti-HIV activities by blocking or downmodulating CCR5 and CXCR4 receptors, that serve as major coreceptors for HIV entry [Cocchi et al., 1995; Verani et al., 1997; Garzino-Demo et al., 1999]. Both the CXC and CC subfamilies have been reported to naturally occur as posttranslational modified isoforms. We initially reported that recombinant CD26/DPPIV was able to cleave RANTES/CCL5 (regulated on activation normal T cell expressed and secreted), IP-10/CXCL10, Eotaxin/CCL24, and MCP-2/CCL8 [Oravecz et al., 1997], resulting in removal of amino-terminal dipeptides from the chemokines. Truncated RANTES(3–68) lost activity on CCR1, but retained CCR5 signaling and HIV blocking capabilities. A number of other chemokines are also substrates for DPPIV including SDF-1/CXCL12 (stromal cell-derived factor), MCP/CCL2, GCP/CXCL6, Mig/CXCL9, GROβ/CXCL2, MDC/CCL22 (macrophage-derived chemokine) [Frohman et al., 1989; Drucker et al., 1997; Ohtsuki et al., 1998; Proost et al., 1998; Shioda et al., 1998; Mentlein, 1999; Proost et al., 2000]. Modification of chemokines by DPPIV proteolytic processing can either inactivate or alter specific receptor interactions [Van Damme et al., 1990; Gong and Clark-Lewis, 1995; Gong et al., 1996; Lusti-Narasimhan et al., 1996; Oravecz et al., 1997; Proost et al., 1998; Boonacker and Van Noorden, 2003].

We have recently reported that human peripheral blood lymphocytes stimulated by cytokines (IL2 and IL12) or phytohemagglutinin (IL2 and PHA) produced an NH_2-terminal truncated form of MIP-1β missing the first two amino acids [Wang et al., 1999; Guan et al., 2001]. MIP-1β is a ligand of CCR5 and can inhibit R5 HIV and gp120 interactions with this receptor. Functional studies of the purified truncated protein revealed that MIP-1β(3–69) retained the ability to down-modulate cell surface expression of the CCR5 and to inhibit CCR5-mediated entry of HIV-1 in T cells. Similar to full length MIP-1β, the truncated protein induced Ca^{2+} signaling through CCR5, but unlike the full-length protein, it gained Ca^{2+} stimulating activity through CCR1 and CCR2b [Guan et al., 2002].

The processing of macrophage inflammatory protein-1β, which contains a putative CD26/DPPIV motif with a proline in second NH_2-terminal position, has not been previously described. In the present study, we identify CD26/DPPIV as a key enzyme in processing of MIP-1β.

MATERIALS AND METHODS

Reagents

Recombinant human MIP-1β was purchased from Sigma (St. Louis, MO); recombinant MIP-1α/CCL3 was obtained from Peprotech (Rocky Hill, NJ); monoclonal antibodies to chemokines were from R&D Systems (Minneapolis, MN).

Cell Culture

Human PBLs and monocytes were isolated by elutriation from normal blood donors and cultured as described [Wang et al., 2001]. PBLs were cultured under 5% CO_2 at 37°C in RPMI 1640 (Life Technologies, Rockville, MD)

supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), IL-2 (150 U/ml) (R & D Systems), and PHA (2 μg/ml) (Sigma). In some experiments, effector peptides {Tat(1–9), W^2Tat(1–9), and TXA2-R(1–9)} or control peptides were added to PBLs culture systems at the concentration of 50 μM on day 1, 2, 3, and 5 for total of 200 μM.

Immunoprecipitation

Cell culture supernatants were incubated at 4°C for 2 h with antibodies to anti-MIP-1α or anti-MIP-1β, and then for an additional 2 h with protein G-Sepharose beads (Amersham Pharmacia Biotech, Uppsala, Sweden). The beads were washed three times with phosphate-buffered saline containing 0.3% Triton X-100, after which proteins were eluted with 25 mM Tris-glycine (pH 3).

Mass Spectrometry

The molecular size of immunoprecipitated proteins was determined by matrix-assisted laser desorption ionization and time-of-flight (MALDI-TOF) mass spectrometry (PerCeptive Biosystems, Boston, MA). α-Cyano-4-hydroxycinnamic acid (Sigma) and recombinant MIP-1α or MIP-1β were used as matrix and internal standards, respectively.

Molecular weights are assigned by the software and may differ between 5 and 6 Da.

CD26 Cleavage

Recombinant human MIP-1β or MIP-1α of 500 nM was incubated with or without human placental CD26 of 250 μU/5 nM (Enzyme Systems, Dublin, CA) in digestion buffer (50 mM Tris, pH 7.5, and 2 mM DTT) for 3, 6, and 24 h at 37°C. Samples were then analyzed using mass spectrometry.

Peptide Synthesis

Tat(1–9) (MDPVDPNIE), its analog W^2Tat (1–9) (MPPVDPNIE) and Tat(46–60) (SYGRKKRRQRRRPPQ), TAX2-R(1-9) (MWPNGSSLG), and control peptide (KDNDYIIPLPDPK) were synthesized using automated fmoc chemistry and HPLC purified by the CBER Core Facility (Bethesda, MD).

Cell Surface Immunofluorescence Staining

After culture in medium containing IL-2 and PHA, PBLs were stained with phycoerythrin-conjugated anti-CD26 monoclonal antibody or an isotype control, and analyzed by flow cytometry as previously described [Wang et al., 1999].

RESULTS

Conversion of lymphocyte-derived MIP-1β to a truncated form correlates with surface expression of CD26 on PBLs. We have reported that human peripheral blood lymphocytes stimulated by cytokines or mitogens produce a NH_2-terminal truncated MIP-1β(3–69) missing the first two amino acids [Guan et al., 2001, 2002]. To investigate whether cell surface expression of CD26 correlates with MIP-1β processing, PBLs were cultured in the presence or absence of IL-2/PHA. The cells were examined for CD26 expression by flow cytometry analysis at various time points (days 1, 3, and 6) and supernatants collected for MIP-1β protein analysis. Baseline levels of CD26 expression were detected before PBLs were exposed to IL-2/PHA. Surface expression of CD26 in PBL steadily increased after IL-2/PHA stimulation with peak level seen at day 6 as shown in Figure 1a. Culture supernatants were then subjected to immunoprecipitation with anti-MIP-1α or anti-MIP-1β, which brings down a complex of these proteins, and the resulting precipitates were analyzed by mass spectrometry. The results revealed that MIP-1β was processed from the intact form (7,826 Da) in the supernatant obtained after 1 day to a mixture of both the intact protein and the (-2) MIP-1β (7,658 Da) after 3 days. The peak at 7,459 Da is the coprecipitated (-4) truncated form of MIP-1α present in the heterodimeric complex as reported previously [Guan et al., 2001]. After incubation of PBLs for 6 days, most of the MIP-1β has been converted to the (-2)MIP-1β form (Fig. 1b). The CD26 expression pattern correlated with MIP-1β proteolytic processing, as evident in the progressive appearance of the truncated form of MIP-1β with increased CD26 expression.

MIP-1β is a substrate for CD26/DPPIV cleavage. MIP-1β is a putative substrate for CD26/DPP IV based on the presence of proline in the second position. However, it is still not clear whether MIP-1β can be cleaved by CD26, since others have reported difficulty showing MIP-1β processing by CD26/DPPIV [Proost et al., 2000]. We found that MIP-1β forms aggregates in solution as evident using native gel electrophoresis, which may contribute to

reduced CD26/DPPIV proteolytic processing (Guan, unpublished observation). To enhance enzyme digestion we pretreated MIP-1β with 2 mM DTT for 1 h and then subjected the protein to CD26/DPPIV enzymatic digestion. Mass spectrometric analysis revealed that 3 to 6-h incubation of recombinant MIP-1β with CD26/DPIV at 37°C in the presence of DTT resulted in partial removal of the NH_2-terminal of Ala-Pro dipeptide, while overnight incubation resulted in complete cleavage of MIP-1β (3–69) (Fig. 2a). Further cleavage was not evident after prolonged incubation with CD26/DPPIV for 48-h. A small amount of MIP-1β(3–69) was generated in the absence of DTT after incubation with CD26/DPPIV for 24 h (Fig. 2b) demonstrating that cleavage can occur in the absence of DTT, but with low efficiency. Recombinant MIP-1α with a penultimate NH_2-terminal serine was not cleaved upon exposure to CD26/DPPIV under the same conditions (Fig. 2a). Our data show that MIP-1β is a substrate for CD26/DPPIV.

MIP-1β/CD26DPP IV processing is blocked by Tat(1–9), W^2-Tat(1–9), and TXA2-R(1–9). Peptides containing the NH_2-terminal amino acid sequence XXP inhibit DPPIV and exhibit similar suppressive effects on the activation of immune cells, as observed using synthetic inhibitors [Herrera et al., 2001]. The HIV-1 transactivator Tat contains this NH_2-terminal XXP motif and Tat(1–86) along with its NH_2-terminal nonapeptide Tat(1–9) (MDPVDPNIE) inhibits DPPIV activity in vitro, and DNA synthesis in T cells [Wrenger et al., 1997; Mrestani-Klaus et al., 2000]. The thromboxane A2 receptor (TXA2-R) also contains a XXP motif and the NH_2-terminal nonapeptide of TXA2-R(1–9) inhibits DPPIV in vitro, and decreases DNA synthesis and IL-2 production from tetanus toxoid-stimulated PBMCs [Wrenger et al., 2000].

To investigate whether CD26/DPPIV is responsible for processing MIP-1β, recombinant MIP-1β was incubated with CD26/DPPIV in the presence of Tat(1–9), W^2Tat(1–9) (a modified Tat peptide), TXA2-R(1–9), Tat(46–60), and a control peptide. Tat(1–9), W^2Tat(1–9), and TXA2-R(1–9) inhibited the processing of recombinant MIP-1β by CD26/DPPIV as shown in Figure 3, while Tat(46–60) and the control peptide had no effect on the processing of MIP-1β. The results demonstrate that CD26/DPPIV is responsible for processing MIP-1β and

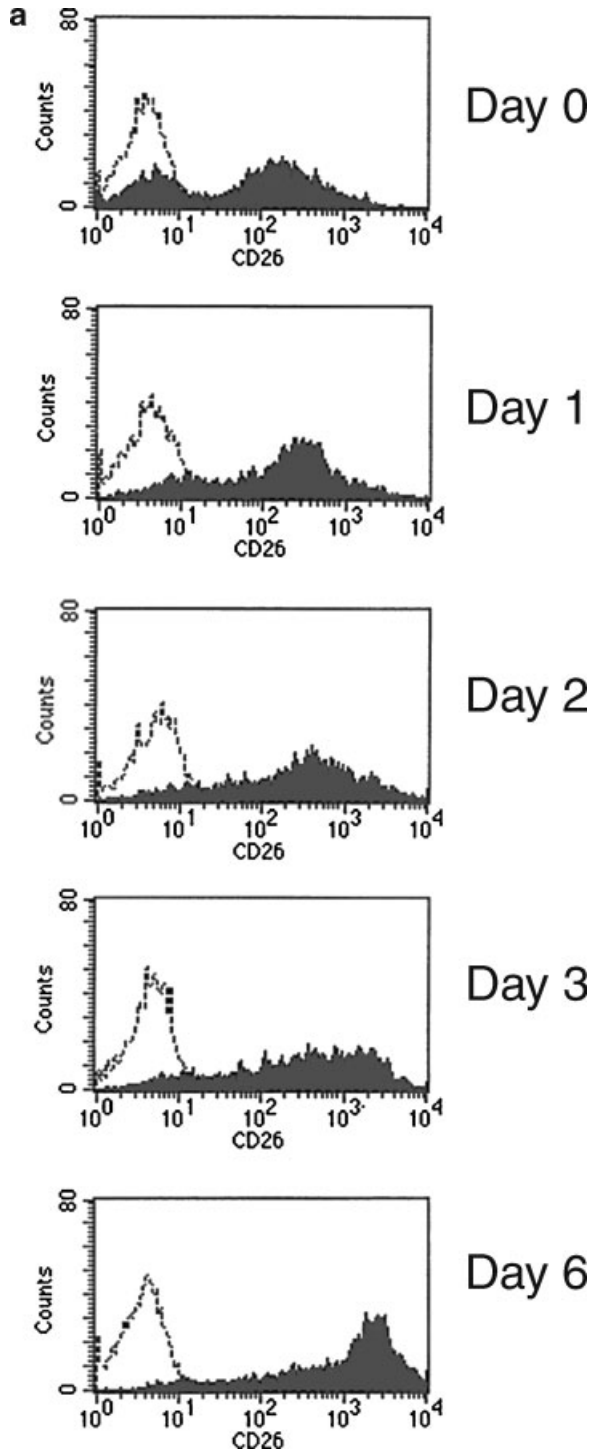

Fig. 1. CD26 expression and mass spectrometric analysis of MIP-1β variants secreted by activated PBLs. PBLs were stimulated with IL-2/PHA for 1, 2, 3, and 6 days, stained with phycoerythrin-labeled monoclonal antibody Ta-1-RD1 to CD26, and analyzed by flow cytometry. **a**: Baseline (day 0) level of CD26 expression for PBLs was determined before addition of IL-2/PHA to the culture. The culture supernatants were collected and subjected to immunoprecipitation with anti-MIP-1β monoclonal antibody. The molecular mass of the precipitated proteins was then determined by mass spectrometry (**b**).

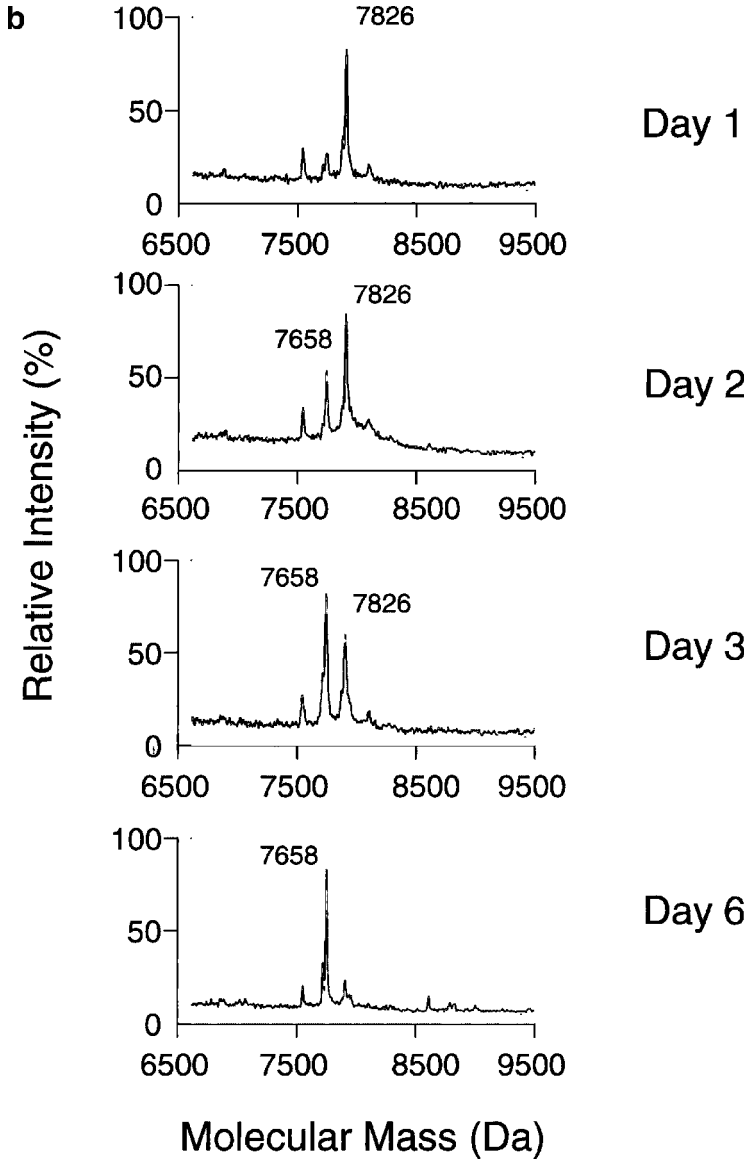

Fig. 1. (*Continued*)

the action of CD26/DPPIV is effectively blocked by the above effector peptides.

Tat(1–9), W^2Tat(1–9), and TXA2-R(1–9) inhibit MIP-1β processing from PBLs. To examine whether these peptides are able to actively inhibit MIP-1β processing in lymphocytes, Tat(1–9), W^2(1–9), TXA2-R, or control peptides were added to PBL cultures in the presence of IL2 and PHA. Culture supernatants and cells were collected on days 1, 3, and 6 for analysis of MIP-1β structure and CD26 expression. Mass spectrometric analysis of immunoprecipitates of culture supernatants prepared with anti-MIP-1β showed that Tat(1–9), W^2Tat(1–9), and TXA2-R(1–9) partially inhibited conversion of full length MIP-1β to MIP-1β(3–69) (7,658). Protein profiles from control (C) peptide and Tat(46–60) peptide (data not shown) treated cultures were similar to untreated cultures, which showed the appearance of predominantly the truncated form of MIP-1β (Fig. 4) by 6 days. In this preparation of cells, the production of the -4 truncated form of MIP-1α of (7,459 Da) was not altered by peptide treatment. Flow cytometry analysis of expression of CD26 on PBLs revealed that Tat(1–9), W^2Tat(1–9), and TXA2-R(1–9) did not reduce CD26 expression, but instead increased cell surface intensity (Fig. 5)

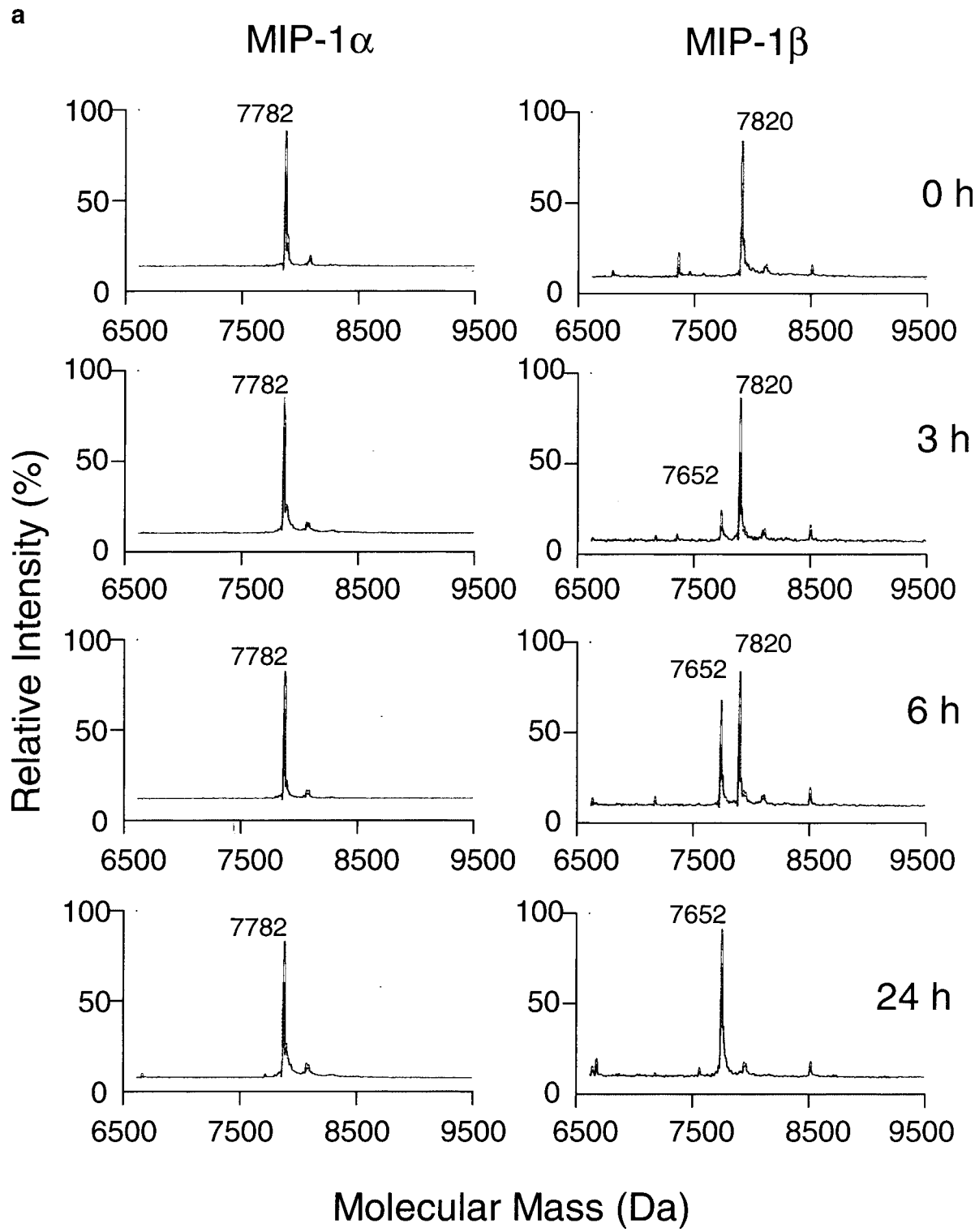

Fig. 2. MIP-1β is cleaved by CD26 DPPIV. **a**: Recombinant MIP-1β(1–69) or MIP-1α was incubated with soluble CD26 for 3, 6, 24 h in the presence of CD26/DPPIV and DTT. **b**: MIP-1β(1–69) was incubated with DTT and CD26 or with CD26 (-DTT) or DTT (-CD26) alone. Samples were subjected to mass spectrometric analysis.

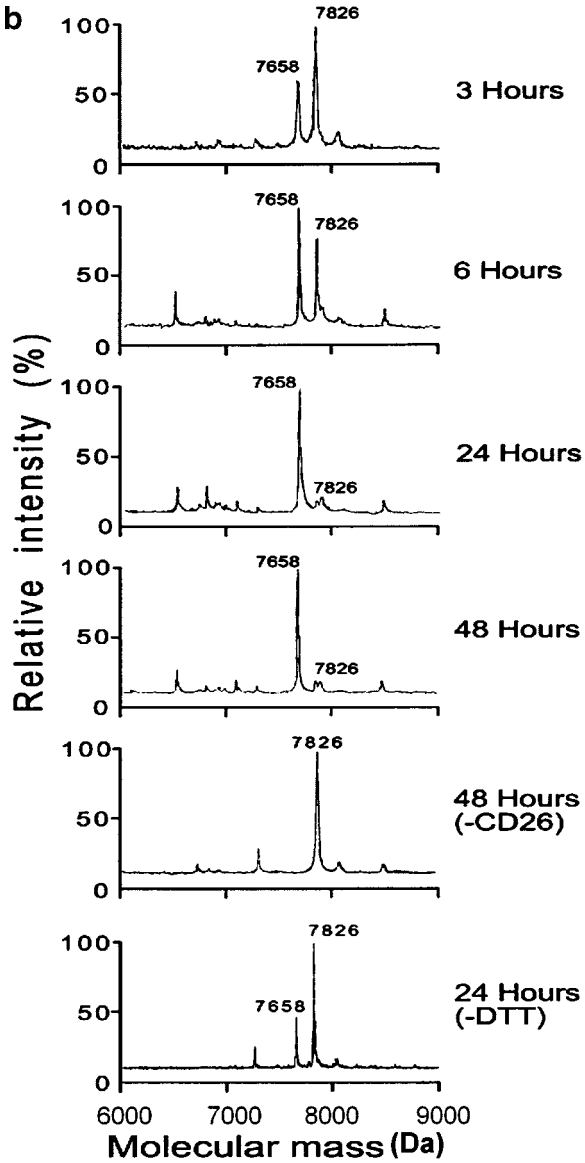

Fig. 2. (*Continued*)

indicating that inhibitors only block DPPIV enzyme activity and not the expression of the protein.

DISCUSSION

Post-translational modifications, such as proteolytic cleavage at NH_2-terminus of polypeptides, have been observed for both CXC and CC chemokines. NH_2-terminal truncation of chemokines has different consequences on biological potency resulting in either increased or decreased specific activity. Activated PBLs release significant quantities of NH_2-terminal truncated MIP-1β variants missing the first two amino acids (Ala-Pro). Although the presence of the NH_2-terminal Ala-Pro in MIP-1β suggested that it could be a substrate for CD26/DPPIV, a resistance to processing of mature MIP-1β by CD26/DPIV has been reported by others [Proost et al., 2000]. In the present study, we examined whether MIP-β could be cleaved by CD26/DPPIV after the penultimate proline as previously shown for the chemokines RANTES, IP-10, eotaxin, MCP-2, GCP-2, SDF-1, and MDC. We observed that incubation of MIP-β with CD26/DPPIV alone generated small amounts of MIP-1β(3–69) while most of the MIP-1β remained intact even after 48 h. Although cleavage was inefficient, this result does verify that DPPIV can cleave MIP-1β directly. This low sensitivity in vitro processing may be due to formation of protein aggregation or occlusion of the amino-terminus of the protein in solution. We found that addition of the reducing agent dithiothreitol potentiated in vitro CD26/DPPIV digestion, resulting in complete conversion of intact MIP-1β into MIP-1β(3–69). The conversion to the truncated form was blocked by Tat(1–9) and its analog, and TAX2-R(1–9) when incubated in vitro with recombinant MIP-1β and DPPIV, and in activated PBLs producing endogenous chemokine.

MIP-1β is a potent chemotactic protein for T cells and macrophages, and inhibits infection of M-Tropic HIV-1 in PBLs by blocking viral entry. We recently reported that MIP-1β(3–69) has altered receptor specificities compared with the full-length form by gaining activity to both CCR1 and CCR2, while retaining potent anti-HIV activity through CCR5 [Guan et al., 2002]. The consequence of CD26/DPPIV mediated truncation of MIP-1β differs from RANTES, MDC, SDF-1α(3–68), where chemokine binding to and signaling through specific receptors were strongly reduced. RANTES lost activity through CCR1, but retained CCR5 functions, while SDF-1α(3–68) lost most of its anti-HIV-1 and signaling activity through CXCR4.

Upregulation of CD26 expression on T lymphocytes has been linked to cell activation and development of memory T cell responses. Expression of CD26 is also associated with Th1 immune responses and Th1 clones have higher amounts of CD26 on the cell surface than Th2 clones [Willheim et al., 1997; Boonaker et al., 2002]. Th1 cells express CCR5, CXCR3, and CXCR6, while Th2 cells express CCR3, CCR4, CCR8, and the prostaglandin D2 chemoattractant receptor CRTh2 [Langenkamp et al.,

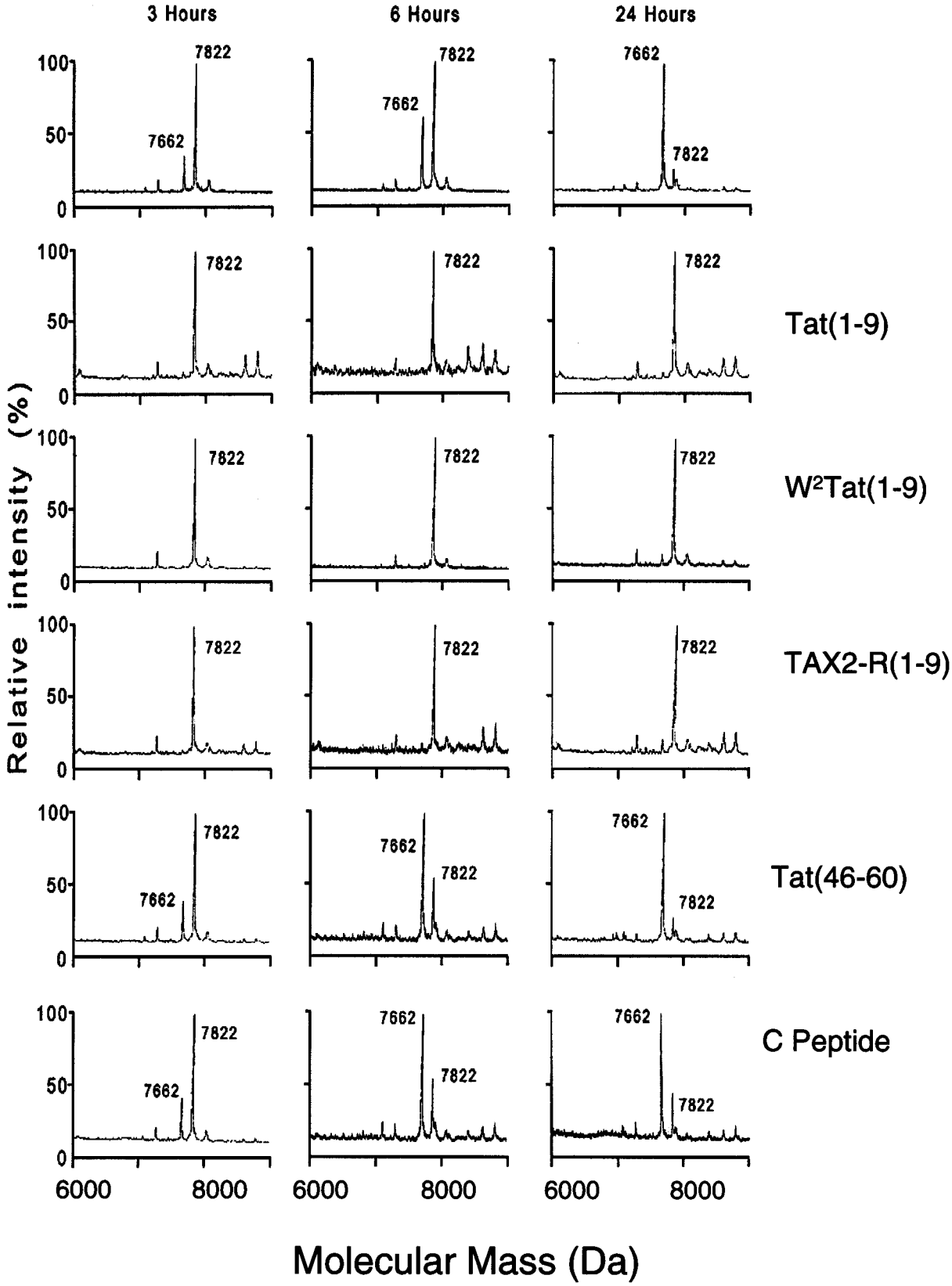

Fig. 3. Tat(1–9) inhibits CD26 processing of MIP-1β. Recombinant MIP-1 β(1–69) was incubated with CD26/DPPIV in the presence or absence of effector peptides or control peptide for 24 h in digestion buffer (50 mM Tris, pH 7.5, and 2 mM DTT) at 37°C. Samples were analyzed by mass spectrometry.

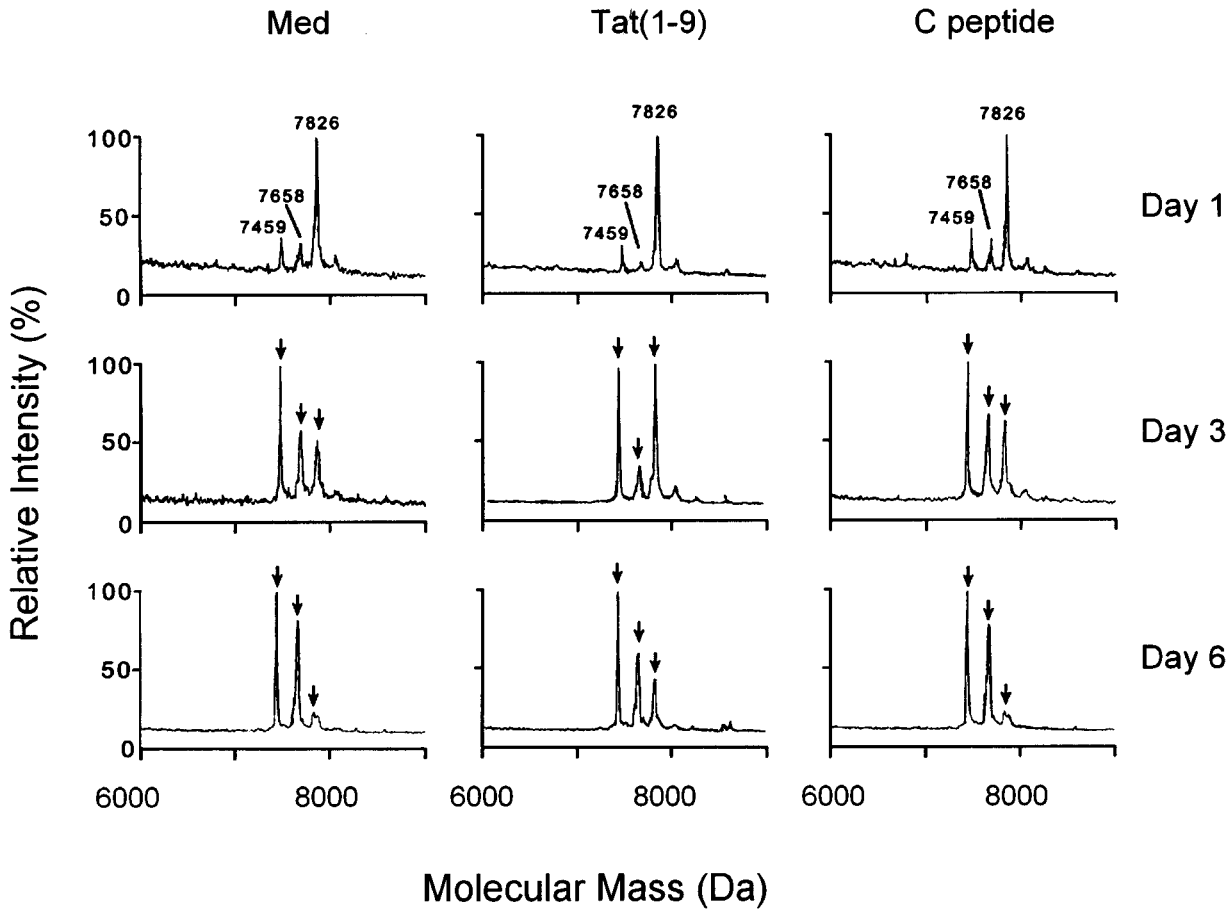

Fig. 4. Natural truncation of MIP-1β from PBLs is blocked by Tat(1–9), W^2Tat(1–9), and TXA2-R. PBLs stimulated with IL2 and PHA in the presence or absence of effector peptides or control peptide. The culture supernatants of PBLs on day 1, 3, and 6 were subjected to immunoprecipitation with anti-MIP-1β monoclonal antibody. The molecular mass of the precipitated proteins was then determined by mass spectrometry.

2003]. Ligands for CCR5, MIP-1β/CCL4, and RANTES/CCL5, retain activity on CCR5 following cleavage by CD26, while ligands for CCR3 and CCR4, eotaxin, and MDC respectively, are inactivated. Th1 cells expressing CD26 may selectively inactivate chemokines that stimulate Th2 recruitment, while maintaining Th1 chemotaxis, and thereby, differentially regulate recruitment of polarized T helper cell type into inflammatory sites. The observed delay in cell expression of CD26 after T cell activation, suggests that cleavage and inactivation of chemokines may function as a feed back mechanism to downmodulate cell recruitment and immune activation. In this respect, it is interesting to note, that MIP-1β/CCL4 has been reported to recruit regulatory T cells that suppress immune responses [Bystry et al., 2001]. Thus, CD26 expression in membrane-bound and soluble forms could affect the course of an inflammatory response by modifying receptor and target cell reactivity of MIP-1β and other chemokines.

Inhibition of the enzymatic activity of DPPIV in vitro, shown in this and other studies, was observed with a number of non-substrate oligopeptides containing an amino-terminal Xaa-Xaa-Pro sequence, e.g., HIV-1 Tat(1–86), Tat(1–9), and Met-IL-2(1–6) [Wrenger et al., 1997; Kahne et al., 1999; Herrera et al., 2001]. The data presented here is the first report on the inhibition of CD26/DPPIV-mediated chemokine (MIP-1β) processing by DPPIV inhibitors, Tat(1–9), or TAX2-R(1–9). Inhibition was observed with both purified DPPIV and in cells expressing CD26, strongly indicating that MIP-1β and probably other chemokines are likely substrates of DPPIV.

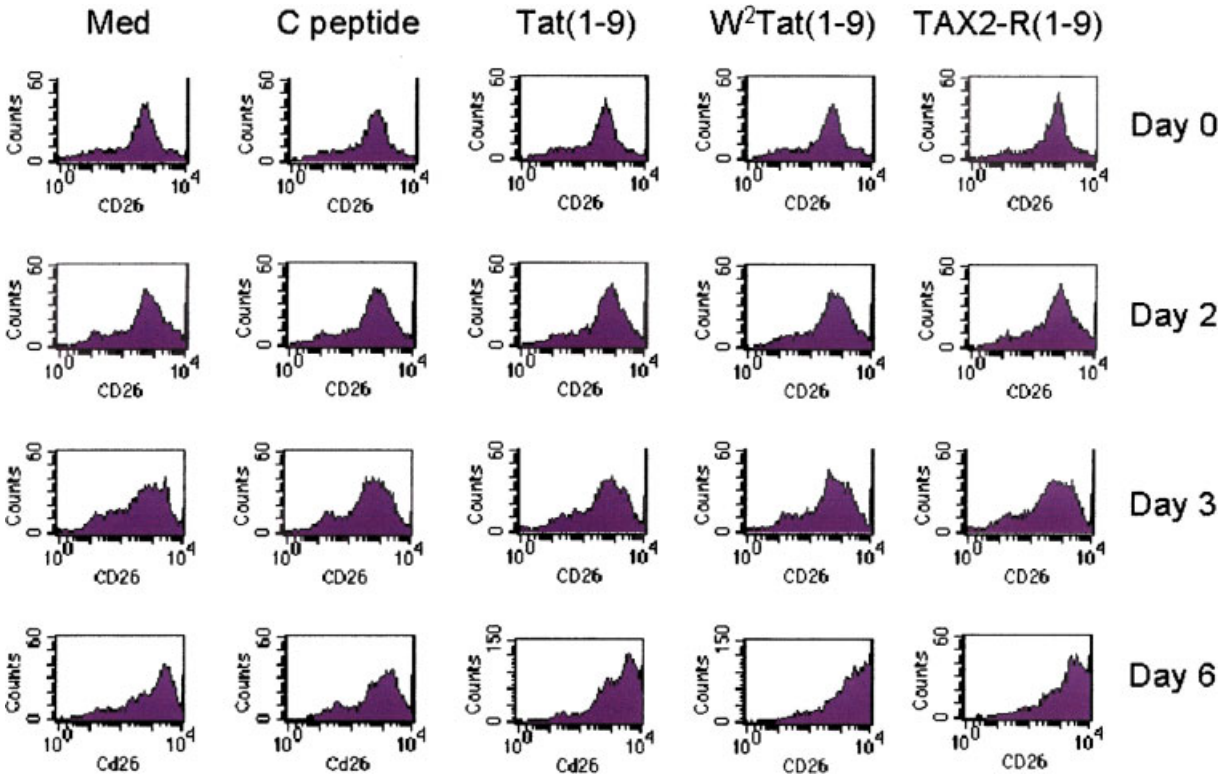

Fig. 5. Effect of putative CD26 inhibitor peptides on cell surface expression of CD26. PBLs stimulated with IL2/PHA in the presence or absence of Tat(1–9), W^2Tat(1–9), or TXA2-R and control peptide. Cell surface expression of CD26 was examined by flow cytometry as indicated on day 2, 3, and 6. Baseline (day 0) level of CD26 expression for PBLs was determined before addition of IL-2/PHA, and control or inhibitor peptides to the culture. [Color figure can be viewed in the online issue, which is available at www. interscience.wiley.com.]

DPPIV inhibitory peptides also had an unexpected effect on cell surface level of CD26. Cells cultured with active peptides showed enhanced expression of CD26. Although the mechanism of this effect is not clear, it may indicate a role for enzyme activity in feedback regulation of CD26 expression, either at the level of RNA transcription, protein translation, or cell surface modulation.

Expression of CD26 is closely correlated with development of immunological memory [Hafler et al., 1989; Morimoto et al., 1989]. Studies have shown that $CD4^+$ cells from HIV-1 infected individuals exhibit a qualitative defect in their ability to generate recall memory responses to antigens. The precise mechanism for loss of memory function has not been fully characterized. It is possible that the binding of Tat to CD26 and inhibition of DPPIV activity may contribute to HIV-associated immune dysfunction either by disrupting costimulatory activity of CD26 or by interfering with chemokine processing. CD26-mediated modification of chemokines (e.g., SDF-1α, SDF-1β) may facilitate HIV-1 replication in T cells and lead to loss of memory T cell function.

This study demonstrates that MIP-1β is a substrate for CD26/DPPIV. The NH_2-terminus plays a critical role for the action of MIP-1β on lymphocytes and monocytes/macrophages. Processing of MIP-1β by CD26/DPPIV generates MIP-1β(3–69) in vitro and in activated T cells resulting in altered biological activity of the chemokine [Guan et al., 2002]. Proteolytic processing of MIP-1β by this dipeptidase may play roles in regulation of T-helper cells and in recruitment and migration of monocyte/macrophages, dendritic cells, and hematopoietic precursors.

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